# Epidemiological Survey of Amoxicillin-Clavulanate Resistance and Corresponding Molecular Mechanisms in *Escherichia coli* Isolates in France: New Genetic Features of *bla*$_{TEM}$ Genes

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Amoxicillin-clavulanate resistance (MIC >16 μg/ml) and the corresponding molecular mechanisms were prospectively studied in *Escherichia coli* over a 3-year period (1996 to 1998) in 14 French hospitals. The overall frequency of resistant *E. coli* isolates remained stable at about 5% over this period. The highest frequency of resistant isolates (10 to 15%) was observed, independently of the year, among *E. coli* isolated from lower respiratory tract samples, and the isolation rate of resistant strains was significantly higher in surgical wards than in medical wards in 1998 (7.8 versus 2.8%). The two most frequent mechanisms of resistance for the 3 years were the hyperproduction of the chromosomal class C β-lactamase (48, 38.4, and 39.7%) and the production of inhibitor-resistant TEM (IRT) enzymes (30.4, 37.2, and 41.2%). By using the single-strand conformational polymorphism–PCR technique and sequencing methods, we determined that 59 IRT enzymes corresponded to previously described IRT enzymes whereas 8 were new. Three of these new enzymes derived from TEM-1 by only one amino acid substitution (Ser130Gly, Arg244Gly, and Asn276Asp), whereas three others derived by two amino acid substitutions (Met69Leu and Arg244Ser, Met69Leu and Ile127Val, and Met69Val and Arg275Gln). The two remaining new IRTs showed three amino acid substitutions (Met69Val, Trp165Arg, and Asn276Asp and Met69Ile, Trp165Cys, and Arg275Gln). New genetic features were also found in *bla*$_{TEM}$ genes, namely, *bla*$_{TEM-1B}$ with either the promoters *Pa* and *Pb*, *P4*, or a promoter displaying a C→G transversion at position 3 of the −35 consensus sequence and new *bla*$_{TEM}$ genes, notably one encoding TEM-1 but possessing the silent mutations originally described in *bla*$_{TEM-2}$ and then in some *bla*$_{TEM}$-encoding IRT enzymes.

Throughout the world, microbiologists are encouraged to survey the antibiotic resistances of major pathogens in order to provide the people in charge of public health with epidemiological data helpful in making recommendations on the best use of antibiotics.

We surveyed amoxicillin-clavulanate resistance in *Escherichia coli* over a 3-year period (1996 to 1998) because amoxicillin-clavulanate is one of the most used antibiotics in France and also because *E. coli* is the major enterobacterial pathogen. In addition to the evolution of amoxicillin-clavulanate-resistant *E. coli* isolates, which was studied both globally and according to different parameters (patient status, wards, and biological specimens), we also analyzed the evolution of the principal mechanisms involved in amoxicillin-clavulanate resistance in *E. coli*. These mechanisms include hyperproduction of the chromosomal class C β-lactamase of *E. coli*, hyperproduction of plasmid-mediated TEM enzymes, production of oxacillinases, and the production of TEM-derived enzymes whose β-lactamase activities are no longer inhibited by clavulanate (inhibitor-resistant TEM [IRT] enzymes) (11–13, 25, 26, 28, 33). However, all these mechanisms which are able to generate amoxicillin-clavulanate resistance in *E. coli* do not generate the same β-lactam cross-resistance. Thus, a knowledge of the evolution of these different mechanisms might lead us to give different recommendations for the choice of antibiotics to treat an infection presumed to be caused by *E. coli*.

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## MATERIALS AND METHODS

**Clinical isolates.** During the same 3 months of the years 1996, 1997, and 1998, the microbiological laboratories of 14 (13 in 1997) French hospitals distributed around the country (seven teaching and seven nonteaching hospitals) collected consecutive and nonrepetitive *E. coli* isolates (only one isolate with a given antibiogram per patient) and filled out a form for each isolate in which the patient status (out-or inpatient), the ward (pediatrics, geriatrics, medicine, surgery, or intensive-care unit) in which each inpatient was hospitalized, and the biological specimen from which each isolate was obtained were specified. The isolates considered resistant to amoxicillin-clavulanate on the basis of the agar disk diffusion method and in accordance with the recommendations of the French Antibiogram Committee (inhibition zone diameter, <14 mm) (1) were sent with their corresponding antibiograms to our laboratory, where we confirmed the resistance and analyzed the underlying mechanisms by molecular methods.

**Susceptibility testing.** Amoxicillin-clavulanate resistance was confirmed for each isolate by the E-test method (AB Biodisk, Dammartin sur Tigeaux, France) performed according to the manufacturer's recommendations. The amoxicillin-clavulanate-resistant isolates were classified in two groups on the basis of the standard antibiogram performed by each laboratory: group I, comprising the isolates displaying an additional resistance to cefpodoxime (inhibition zone diameter, <21 mm) and a reduced susceptibility to cefoxitin (inhibition zone diameter, <22 mm) (1), and group II, comprising the isolates fully susceptible to these two antibiotics. The isolates of group I were considered to be hyperproducing their chromosomal class C β-lactamase and not to be producing plasmid-mediated AmpC-like enzymes on the basis of the conserved susceptibility to broad-spectrum cephalosporins or aztreonam (3, 6, 15), and no further molecular analysis was made in this study to characterize this mechanism of resistance.

**Molecular detection of TEM, IRT, and OXA-1 enzymes.** As previously described, two specific primers of *bla*$_{TEM}$ genes were used to amplify the entire gene and its promoter region and two other primers specific for the gene encoding OXA-1 were used to amplify an internal 609-bp fragment of this gene. When the PCR was positive for the *bla*$_{TEM}$ gene, pairs of primers designed to obtain three overlapping fragments were used to carry out the single-strand conformational polymorphism (SSCP)–PCR, as we have previously demon-

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TABLE 1. Frequencies of amoxicillin-clavulanate (AMC)-resistant *E. coli* isolates by patient and hospital characteristics

| Parameter | No. (%) of AMC-resistant *E. coli* isolates | | |
|---|---|---|---|
| | 1996 | 1997 | 1998 |
| Children | 6 (3.8) | 5 (3.0) | 9 (5.1) |
| Adults | 78 (5.5) | 75 (5.7) | 66 (4.6) |
| Outpatients | 7 (2.5)[a] | 7 (2.7)[a] | 10 (3.4) |
| Inpatients | 81 (6.1)[a] | 73 (6.0)[a] | 65 (5.0) |
| Teaching hospitals | 42 (5.6) | 43 (5.5) | 45 (6.0) |
| Nonteaching hospitals | 46 (5.3) | 37 (5.3) | 30 (3.5) |

[a] Significant difference between the frequencies of AMC-resistant *E. coli* isolated in outpatients and inpatients in 1996 ($P = 0.01$) and 1997 ($P = 0.03$).

TABLE 2. Frequencies of amoxicillin-clavulanate (AMC)-resistant *E. coli* isolates by wards

| Ward | No. (%) of AMC-resistant *E. coli* isolates | | |
|---|---|---|---|
| | 1996 | 1997 | 1998 |
| Intensive-care units | 8 (6.0) | 7 (6.5) | 8 (6.7) |
| Surgery | 24 (9.0) | 13 (5.5) | 19 (7.8)[b] |
| Medicine | 27 (4.5) | 40 (7.0)[a] | 16 (2.8)[a,b] |
| Geriatrics | 9 (7.3) | 6 (5.0) | 10 (7.0) |

[a] Significant decrease between the frequencies of AMC-resistant *E. coli* in 1998 and 1997 in medical wards ($P = 0.008$).
[b] Frequency of AMC-resistant *E. coli* was significantly lower in medical wards than in surgical wards in 1998 ($P = 0.001$).

strated (34). Briefly, each $^{32}$P-labeled amplified fragment was submitted to electrophoresis on a native polyacrylamide gel after heat and formamide denaturation. The electrophoretic mobility of each fragment was compared to that of the corresponding fragment of the reference $bla_{TEM}$ genes: $bla_{TEM-1A}$, $bla_{TEM-1B}$, and $bla_{TEM-2}$. The $bla_{TEM}$ genes whose three fragments comigrated with the corresponding fragments of reference $bla_{TEM}$ genes were assessed as TEM-encoding genes, whereas those whose fragment 1, 2, or 3 did not comigrate with the corresponding fragments of the reference genes were assessed as $bla_{TEM}$ gene derivatives and subsequently sequenced.

**Nucleotide sequence.** PCR products for sequencing were prepared under the standard conditions described above. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Courtaboeuf, France) following the manufacturer's recommendations. The nucleotide sequences of purified PCR fragments were determined with an automated cycle-sequencing system on a Perkin-Elmer R377 sequencer and the same primers used for PCR (34).

**Statistical analysis.** The differences between proportions were evaluated by a chi-square test at the 5% level of significance.

**Nucleotide sequence accession numbers.** The nucleotide sequence data reported here for $bla_{TEM-76}$, $bla_{TEM-77}$, $bla_{TEM-78}$, and $bla_{TEM-79}$ have been submitted to GenBank and have been assigned accession no. AF 190694, AF 190695, AF 190693, and AF 190692, respectively.

## RESULTS

**Distribution of *E. coli* clinical isolates.** One thousand six hundred twenty and 1,606 *E. coli* isolates were collected in 1996 and 1998, respectively; only 1,483 were collected in 1997 because one of the 14 laboratories was not able to participate in the study that year. There were no statistical differences in the distributions of these isolates according to the different parameters except for the deep-pus specimens, whose frequency was significantly ($P < 0.001$) higher in 1998 (10.3%) than in 1996 (6.7%) and 1997 (6.8%). Each year, the teaching hospitals provided approximately one-half of the isolates and the nonteaching hospitals provided the other half. Each year, 10% of the isolates came from children and 90% came from adults, and 18 and 82% were from outpatients and inpatients, respectively. About 8% of the isolates were obtained from adult patients hospitalized in intensive-care units, 8% were from patients in geriatrics, 16% were from patients in surgery, and 38% were from patients in medical wards. The great majority of isolates (80% in 1996 and 1997 and 75.5% in 1998) were urinary tract isolates, while 9 and 3% were isolated from blood and lower respiratory tract secretions, respectively.

**Epidemiology of *E. coli* isolates resistant to amoxicillin-clavulanate.** Amoxicillin-clavulanate resistance, first determined by the agar disk diffusion method (inhibition zone diameter, <14 mm) for 79 isolates in 1996, 78 in 1997, and 68 in 1998, was subsequently confirmed by the E-test method (MIC >16 µg/ml) (1). Thus, 4.8% of *E. coli* isolates collected in 1996, 5.2% of those collected in 1997, and 4.2% of those collected in 1998 were resistant, but these frequency differences were not statistically significant.

As indicated in Table 1, there was no significant evolution of the frequency of amoxicillin-clavulanate-resistant *E. coli* iso-

lates in children, adults, out- and inpatients, or teaching and nonteaching hospitals. The comparison of the frequency of amoxicillin-clavulanate-resistant isolates which was made each year among all the different patient and hospital categories showed that the frequency of these isolates was significantly higher for inpatients than for outpatients only in 1996 ($P = 0.01$) and 1997 ($P = 0.03$).

Regarding the rate of amoxicillin-clavulanate-resistant *E. coli* isolates studied in the different wards (Table 2), it was shown that there was a significant evolution of this rate only in medical wards and only for the years 1997 and 1998. In fact, in this type of ward, the frequency of amoxicillin-clavulanate-resistant *E. coli* isolates significantly decreased, from 7% in 1997 to 2.8% in 1998 ($P = 0.008$). The comparative analysis of frequencies between different wards for each year showed that there was no significant difference, except in 1998 between medical and surgical wards, in which the frequencies of amoxicillin-clavulanate-resistant *E. coli* isolates were 2.8 and 7.8%, respectively ($P = 0.001$).

Irrespective of the year in question, the highest frequency of amoxicillin-clavulanate-resistant *E. coli* isolates was observed among the *E. coli* strains isolated from lower respiratory tract samples (Table 3). In 1997 and 1998, this prevalence was significantly higher than those determined for the other samples (urine, blood samples, and deep pus). For the same type of sample, the frequency of amoxicillin-clavulanate-resistant *E. coli* isolates varied over the 3 years, but not significantly, except for blood samples, for which the frequency was significantly higher in 1996 (7.3%) than in 1998 (1.4%) ($P = 0.04$).

**Mechanisms of resistance to amoxicillin-clavulanate.** Among the 225 amoxicillin-clavulanate-resistant *E. coli* isolates collected over the 3-year period, 98 isolates which displayed a coresistance to amoxicillin-clavulanate and cefpodoxime and a

TABLE 3. Frequencies of amoxicillin-clavulanate (AMC)-resistant *E. coli* isolates by sample

| Sample | No. (%) of AMC-resistant *E. coli* isolates | | |
|---|---|---|---|
| | 1996 | 1997 | 1998 |
| Lower respiratory tract | 5 (9.4) | 6 (14.6)[b] | 7 (15.2)[b] |
| Urine | 67 (5.2) | 67 (5.6)[b] | 52 (4.3)[b] |
| Blood | 8 (6.0)[a] | 4 (3.1)[b] | 2 (1.4)[a,b] |
| Deep pus | 8 (7.3) | 3 (2.9)[b] | 8 (4.8)[b] |

[a] Significantly lower frequencies of AMC-resistant *E. coli* isolates in blood in 1998 than in 1996 ($P = 0.04$).
[b] Significantly higher frequencies of AMC-resistant *E. coli* isolates in lower respiratory tract samples than in each other type of sample in 1997 and 1998; urine (1997, $P = 0.03$; 1998, $P = 0.04$), blood (1997, $P = 0.01$; 1998, $P = 0.0007$), and deep pus (1997, $P = 0.02$; 1998, $P = 0.02$).

reduced susceptibility to cefoxitin were considered to be hyperproducing their chromosomal class C β-lactamase. However, for 47 (48%) of them, the $bla_{TEM}$ PCR was positive, and TEM enzyme production was demonstrated in 41 of the cases on the basis of SSCP-PCR experiments, as the three amplified $bla_{TEM}$ fragments of the corresponding genes comigrated with those of reference $bla_{TEM}$ genes. For six isolates which displayed SSCP-PCR profiles different from those of the reference $bla_{TEM}$ genes, the nucleotide sequences of the corresponding $bla_{TEM}$ genes showed the presence of either $bla_{TEM}$-like genes ($n = 3$) or $bla_{TEM}$ genes encoding IRT enzymes ($n = 3$). The $bla_{TEM}$ gene of strain 602042 differed from $bla_{TEM-1B}$ by the silent mutation G162T, whereas that of strain 710020 resembled $bla_{TEM-1A}$ except for the replacement of cytosines 436 and 913 by thymidines (Table 4). The third $bla_{TEM}$-like gene, found in strain 606160, was more closely related to $bla_{TEM-2}$, although the nucleotide mutation leading to the amino acid substitution Glu39Lys was absent and the promoter region was different, showing thymidine 32 replaced by cytosine and guanine 162 replaced by thymidine (Table 4). The amino acid sequences deduced from the nucleotide sequences of the $bla_{TEM}$ genes of strains 610087, 710085, and 609046 showed that the two first strains produced IRT-2 (TEM-30) whereas the third produced a new TEM derivative in which there was only one amino acid substitution, namely, Asn276Asp (Table 4).

Among the 127 isolates resistant to amoxicillin-clavulanate but susceptible to cefpodoxime and cefoxitin, 9 displayed an OXA-1-positive PCR, 117 had a TEM-positive PCR, and 1 had both OXA-1- and TEM-positive PCRs.

For 32 out of the 118 $bla_{TEM}$ PCR products, the electrophoretic mobilities of the three amplified fragments were identical to those of the reference $bla_{TEM}$ genes.

A particular SSCP-PCR profile was observed for 17 out of the 118 $bla_{TEM}$ PCR products, meaning that the profile of each fragment was composed of four bands instead of two (Fig. 1). As two of these bands comigrated with those of reference $bla_{TEM}$ genes and two did not, this profile was thought to have resulted from the coamplification of $bla_{TEM}$ and $bla_{TEM}$ derivative genes.

The nucleotide sequence determined for the remaining 69 $bla_{TEM}$ PCR products displaying SSCP-PCR profiles different from those of reference $bla_{TEM}$ genes showed that four of them were $bla_{TEM}$-like genes (Table 4) and 65 were $bla_{TEM}$ gene derivatives. Three of the $bla_{TEM}$-like genes were $bla_{TEM-1B}$-like genes, each demonstrating a singular silent mutation to distinguish it from $bla_{TEM-1B}$: C32T in strain 714028, A346G in strain 801148, and C141G in strain 805009 (Table 4). The fourth $bla_{TEM}$-like gene was a $bla_{TEM-1C}$ gene which differed from $bla_{TEM-1A}$ by the mutation C436T.

The amino acid sequences deduced from the nucleotide sequences of the remaining 65 $bla_{TEM}$ genes showed that 57 isolates produced previously described IRT enzymes: 15 isolates produced IRT-2 (TEM-30; one of these also produced an OXA-1 enzyme), 14 produced IRT-11 (TEM-40), 9 produced IRT-4 (TEM-35), 6 produced IRT-8 (TEM-37), 4 produced IRT-7 (TEM-36), 3 produced IRT-6 (TEM-34), 3 produced IRT-10 (TEM-39), 1 produced IRT-5 (TEM-33), 1 produced IRT-1 (TEM-31), and 1 produced IRT-9 (TEM-38).

Eight isolates produced IRT enzymes which have not yet been described (Table 4). Three new IRT enzymes derived from TEM-1 by one amino acid substitution: Ser130Gly (strain 811020; TEM-76), Arg244Gly (strain 803013; TEM-79), and Asn276Asp (strains 614078 and 609046; TEM-84). Three other new IRT enzymes derived from TEM-1 by two amino acid

TABLE 4. Nucleotide mutations and amino acid substitutions in $bla_{TEM}$-like genes and new IRT-encoding genes in comparison with $bla_{TEM-1A}$, $bla_{TEM-1B}$, $bla_{TEM-1C}$, and $bla_{TEM-2}$ genes

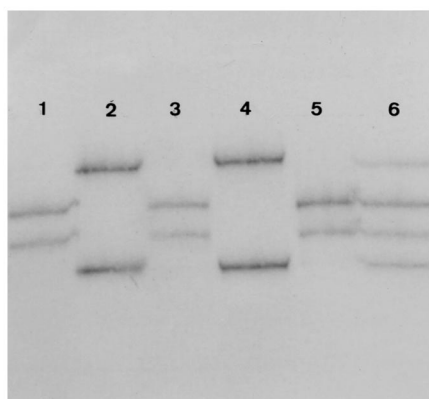| Strain | $bla_{TEM}$ | 32 | 141 (−35) | 162 (−10) | 175 | 226 (6) | 317 (39) | 346 (48) | 407 (69) | 436 (78) | 581 (127) | 590 (130) | 604 (134) | 682 (160) | 695 (165) | 697 (165) | 913 (237) | 925 (242) | 929 (244) | 1020 (275) | 1022 (276) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1A | | C | C | G | A | C | C (Gln) | A | A (Met) | C | A (Ile) | A (Ser) | | | T (Trp) | G (Trp) | C | | C (Arg) | G (Arg) | A (Asn) | 36 |
| 1B | | C | G | G | G | | A | | A | | | | | | | | | | | | | 18a |
| 1C | | | | | | | | | | T | | | | | | | T | | | | | 18 |
| 2 | | T | | | G | | A (Lys) | | A | | | | | | | | | | | | | 18a |
| 602042 | 1B-like | | | T | | | | | | | | | | | | | | | | | | |
| 714028 | 1B-like | T | | | | | | | | | | | | | | | | | | | | |
| 805009 | 1B-like | | G | | G | T | | G | | T | | | | | T | | | | | | | |
| 710020 | 1D | | | | G | T | | G | | T | | | | | T | | T | | | | | |
| 801148 | 1E | | | | G | | | G | | T | | | | | T | | | | | | | |
| 606160 | 1F | | T | T | G | | | | | T | | | | | T | | | | | | | |
| 609046 | 84 | | | T | G | T | | G | | T | | G | | | T | | | A | A | A | G (Asp) | |
| 614078 | 84 | | | T | G | T | | G | | T | | G | | | T | | | A | A | A | G (Asp) | |
| 811020 | 76 | | | G | G | T | | G | | T | | G (Gly) | G | G | T | | | A | A | A | | |
| 803013 | 79 | | | G | G | T | | G | | T | | G (Gly) | | | T | T | | | A | G (Gly)／A (Ser) | G (Asp)／A (Asn) | |
| 801009 | 77 | | T | G | G | T | | G | C (Leu) | T | | | C (Leu) | | T | T | | | A | G (Gly)／A (Asp) | G (Asp) | |
| 601105 | 81 | | T | G | | T | | | C (Leu) | T | | | C (Leu) | | | T | | | | A (Gln) | | |
| 614024 | 82 | T | T | | | T | | | G (Val) | T | | G (Val) | C (Val) | | | T | | | | A (Gln) | | |
| 801155 | 78 | T | T | | | T | | | G (Val) | T | | G (Val) | G (Val) | | | T | | A | | A (Gln) | | |
| 813147 | 83 | | | T | G | T | G | G | T (Ile) | T | | T (Ile) | T (Ile) | C | T | C | C | T (Cys) | A | A (Gln) | G (Asp) | |

FIG. 1. SSCP profiles of fragment 3 of $bla_{\text{TEM}}$ genes expressed by four clinical *E. coli* isolates and of reference genes $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-2}}$. As the nucleotide sequences of $bla_{\text{TEM-1A}}$ and $bla_{\text{TEM-1B}}$ are identical for fragment 3, there is only one SSCP profile for the two genes, which is shown in lane 3. The SSCP profile of fragment 3 of the reference $bla_{\text{TEM-2}}$ gene is shown in lane 4. Two clinical isolates (lanes 1 and 5) expressed a $bla_{\text{TEM}}$ gene whose fragment 3 comigrates with that of $bla_{\text{TEM-2}}$. The migration profile of fragment 3 of the $bla_{\text{TEM}}$ gene expressed by a third clinical isolate (lane 2) differs slightly from that of $bla_{\text{TEM-2}}$ (lane 4). In lane 6 is shown a migration profile consisting of four bands. As two of these bands comigrate with those of $bla_{\text{TEM-1}}$ and the other two do not comigrate with those of any reference genes, this profile is thought to result from the coamplification of $bla_{\text{TEM-1}}$ and a $bla_{\text{TEM}}$ derivative.

substitutions: Met69Leu and Arg244Ser (strain 801009; TEM-77), Met69Leu and Ile127Val (strain 601105; TEM-81), and Met69Val and Arg275Gln (strain 614024; TEM-82). The last two new IRT enzymes derived from TEM-1 by three amino acid substitutions: Met69Val, Trp165Arg, and Asn276Asp (strain 805155; TEM-78) and Met69Ile, Trp165Cys, and Arg275Gln (strain 813147; TEM-83).

**Evolution of the mechanisms involved in amoxicillin-clavulanate resistance.** As indicated in Table 5, the frequency of *E. coli* isolates whose amoxicillin-clavulanate resistances were considered to be related to the hyperproduction of their chromosomal class C β-lactamase decreased over the 3-year period (48% in 1996 and 39.7% in 1998), whereas the frequency of those whose amoxicillin-clavulanate resistances were presumed to be related to the production of IRT enzymes increased (30.4% in 1996 and 41.2% in 1998). However, this opposite evolution was not significant as was also the case for the decrease in TEM-producing isolates (15% in 1996 and 10.3% in 1998) and the increase in OXA-1-producing isolates (3.8% in 1996 and 7.3% in 1998). The association of two mechanisms responsible for amoxicillin-clavulanate resistance, such as OXA-1 and IRT enzymes or IRT enzymes and presumed hyperproduced class C β-lactamase, remained rare (Table 5).

## DISCUSSION

Amoxicillin-clavulanate, which was first produced commercially almost 20 years ago, continues to be a contributing antibiotic to overcome the problem of class A β-lactamase-producing bacteria, especially fastidious bacteria, such as *Haemophilus influenzae* and *Moraxella catarrhalis*. Such a feature can certainly explain why amoxicillin-clavulanate is so intensively prescribed, and sometimes abused, to empirically treat otitis, sinusitis, and respiratory tract infections (19). In the case of the normally amoxicillin-susceptible enterobacteria, particularly *E. coli*, the emergence of isolates less susceptible to amoxicillin-clavulanate was noted as early as 1987 among the

amoxicillin-resistant isolates (25). Since then, several studies which evaluated the antibiotic susceptibilities of enterobacteria in different European countries have shown that the frequency of *E. coli* with reduced susceptibility to amoxicillin-clavulanate has not exceeded 25 to 30% in hospitals (8, 17, 24, 27, 29, 31; M. H. Nicolas-Chanoine, J. Sirot, and H. Chardon, *Abstr.* 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 2253, 1999) and 10% in the community (16) for the last 10 years. Nevertheless, some studies reported frequencies of nonsusceptible *E. coli* isolates of 40% (21, 27), whereas others reported frequencies as low as 5% (35). As recently pointed out by Simpson et al., differences in amoxicillin-clavulanate susceptibility among the *E. coli* isolates of different countries can result from real localized resistance problems but also from methodological differences in susceptibility testing and breakpoint criteria (32). Moreover, the group of nonsusceptible isolates includes intermediate as well as resistant isolates, and the classification of isolates into these two subcategories also depends on methodological criteria. Our study focused on *E. coli* strains for which the amoxicillin-clavulanate MIC was higher than 16 µg/ml, i.e., on *E. coli* considered to be resistant to amoxicillin-clavulanate irrespective of the methodology used (32).

To our knowledge, this is the first study which has observed the evolution of the amoxicillin-clavulanate susceptibility of *E. coli* over a 3-year period in a large number of hospitals by taking into account different criteria, such as patient characteristics, hospital status, medical specialties, and sample origins.

During the 3-year period of our study (1996 to 1998), the overall frequency of amoxicillin-clavulanate-resistant *E. coli* remained stable at about 5%. A similar observance of a stable frequency was also made by Stapleton, who conducted a survey from 1990 to 1994 but described much lower resistance rates (35). The percentage of 5% resistant isolates was found, in our study, for urinary tract isolates of *E. coli*, and this is quite a bit lower than that of Henquell et al., who reported about 25% resistant isolates obtained from a single hospital and 10% obtained from private laboratories in Clermont Ferrand, France, in 1993 (21). As their study included only one hospital, this difference could be attributed to the local spread of *E. coli* clones.

The highest frequencies of amoxicillin-clavulanate-resistant *E. coli* isolates were observed in samples from the lower respiratory tract. These frequencies, which increased from 9.4% in 1996 to 14.6% in 1997 and to 15.2% in 1998, were significantly higher than those in all other samples. Such an observation should be taken into account by clinicians in charge of patients with chronic lung diseases who are hospitalized for acute lower

TABLE 5. Distribution of amoxicillin-clavulanate resistance mechanisms in 225 *E. coli* isolates by year

| Mechanism[a] | No. (%) of *E. coli* isolates | | | |
|---|---|---|---|---|
| | 1996 | 1997 | 1998 | Total |
| CASE (± TEM) | 38 (48) | 30 (38.4) | 27 (39.7) | 95 (42.2) |
| TEM | 12 (15) | 17 (21.8) | 7 (10.3) | 36 (16) |
| OXA-1 | 3 (3.8) | 1 (1.3) | 5 (7.3) | 9 (4) |
| IRT (± TEM) | 24 (30.4) | 29 (37.2) | 28 (41.2) | 81 (36) |
| CASE + IRT | 2 (2.5) | 1 (1.3) | 0 | 3 (1.3) |
| OXA-1 + IRT | 0 | 0 | 1 (1.5) | 1 (0.4) |
| Total | 79 (100) | 78 (100) | 68 (100) | 225 (100) |

[a] CASE, cephalosporinase; OXA-1, oxacillinase-1.

respiratory tract infections because amoxicillin-clavulanate is one of the primary drugs recommended for the treatment of such patients (2, 22). With regard to the different medical specialties, it is interesting to note that *E. coli* isolates obtained from patients in surgery wards, independent of the sample origin, were significantly more often resistant to amoxicillin-clavulanate than those from patients in medical wards in 1998. Those surgeons who are in charge of patients with abdominal problems should be aware of this result, because amoxicillin-clavulanate, which is indicated in France for the treatment of intra-abdominal infections, is one of the most prescribed antibiotics for its concomitant activity against enterobacteria and anaerobes.

In addition to the clinical epidemiology of amoxicillin-clavulanate-resistant *E. coli*, we also studied the corresponding mechanisms of resistance and their evolution. Although relevant for assessing the adaptation of bacteria to antibiotic pressure, studies of the molecular epidemiology of mechanisms of resistance remain rare. Henquell et al., who studied the mechanisms of resistance to amoxicillin-clavulanate of urinary tract *E. coli* isolates exclusively, found that TEM production (48%) was the first mechanism and IRT production (27.5%) the second mechanism for *E. coli* isolated from hospitalized patients and the converse (34% TEM and 45% IRT production) for *E. coli* isolates collected by private laboratories in Clermont Ferrand, France (21). In our study, we found quite a different distribution of mechanisms, showing presumed hyperproduction of the class C β-lactamase as the predominant mechanism (48%) in 1996, followed by IRT production (30.4%), while both mechanisms were found to be equivalent in 1997 (38.4 and 37.2%) and 1998 (39.7 and 41.2%). The fact that IRT production tended to increase could appear paradoxical, because this mechanism is linked to a narrower spectrum of β-lactam hydrolysis than that of class C β-lactamase production. However, it might reflect the antibiotic selective pressure by penicillin molecules, which have been widely used over the last 10 years. Amoxicillin-clavulanate resistance due to the pure production of TEM enzymes was, irrespective of the study year, always at a lower rate than that found by Henquell et al. (21). However, in both studies the frequencies of OXA-1-producing *E. coli* remained low. We confirm in this study the previously described relevance of the SSCP-PCR method for screening and differentiating genes encoding TEM- and TEM-derived β-lactamases. By using this method, we were able to detect new $bla_{TEM}$-like genes and new IRT-encoding $bla_{TEM}$ genes. Regarding the new $bla_{TEM}$-like genes, we found $bla_{TEM-1B}$ genes with either the strong promoters $Pa$ and $Pb$ (C32T), described by Chen (14) and initially present in $bla_{TEM-2}$, or the strong promoter (G162T) recently designated $P4$ by Goussard et al. (18). Given that $bla_{TEM-1B}$-derived genes, such as $bla_{TEM-30}$ (10), $bla_{TEM-33b}$ (18), and $bla_{TEM-38}$ (34), possess promoters $Pa$ and $Pb$, we consider that we have discovered the ancestral $bla_{TEM-1B}$ gene of these derivatives. A third $bla_{TEM1B}$-like gene also displays modifications in the promoter, namely, the mutation C141G located at position 3 in the −35 consensus of the Pribnow box, which is a new genetic feature in the promoters of $bla_{TEM}$ genes. A fourth $bla_{TEM}$ gene is similar to $bla_{TEM-1C}$ (18) except for the mutation C913T, whereas a fifth is similar to $bla_{TEM-1B}$ except for the mutation A346G, previously described in the $bla_{TEM-2}$ gene. We suggest designating these two genes $bla_{TEM-1D}$ and $bla_{TEM-1E}$, respectively. The last $bla_{TEM}$-like gene we described is completely new. In fact, it possesses all the silent mutations observed in the $bla_{TEM-2}$ gene in comparison with the $bla_{TEM-1A}$ gene but not the amino acid substitution Gln39Lys, and it displays the strong promoter $P4$ instead of $Pa$

and $Pb$ as in the $bla_{TEM-2}$ gene. As these silent mutations and this promoter are shared by several $bla_{TEM}$ genes encoding IRT enzymes (TEM-33c, -34, -35, -36, -37, -39, and -45) (9, 10, 18, 34), we think we have identified the ancestor of these derivatives, and we therefore suggest calling this gene $bla_{TEM-1F}$.

Our study has also contributed to the discovery of eight new IRT enzymes. One has a mutation only at position 276 which has been previously described only in an in vitro mutant (30). A second resembles TEM-59, as it possesses the amino acid substitution Ser130Gly, but derived from TEM-1 and not from TEM-2 (5, 23). A third differs from TEM-30, -31, -41, and -51 by a glycine at position 244 (7).

Three new enzymes show an association of two amino acid substitutions. The first combines two substitutions already described individually in TEM-33 and TEM-30 (4, 20). The second includes a known substitution and a new substitution in terms of its position. The third resembles TEM-45 (9).

The two remaining new IRT enzymes display three amino acid substitutions. One of them corresponds to a new combination of 3-amino-acid substitutions, whereas the other resembles TEM-39 (20).

In conclusion, this study, which permitted a large-scale evaluation of the frequencies of amoxicillin-clavulanate-resistant *E. coli* isolates in hospitals, also permitted the discovery of new $bla_{TEM}$ genes as well as new $bla_{TEM}$ derivatives.

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